The New Biomedical Technology

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New methods for studying the genetic information of humans in health and disease are emerging from basic science laboratories. Because these approaches are yielding fundamental insights for diagnosing and treating disease, it is important that practitioners begin to understand these methods and how they are used. Methods for genetic analysis using recombinant DNA techniques consist of isolation, separation, propagation in microorganisms and molecular hybridization of DNA. The study of RNA allows determination of gene expression. These methods are being used to understand cancer, identify hereditary illness, produce pharmaceuticals and diagnose common clinical problems, such as infectious diseases.

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A great discovery is a fact whose appearance in science gives rise to shining ideas, whose light dispels many obscurities and shows us new paths.

CLAUDE BERNARD[1]

In the past ten years there have been remarkable advances in techniques for the study of the genetic information of humans and other organisms. Various terms are used to describe the general methodology of such techniques, including "recombinant DNA research," "genetic engineering" and "cloning." The time has come when these tools of the basic laboratory are immediately relevant to clinical medicine. These methods are not only being used to study disease in an entirely new light, but they are providing more precise diagnostic techniques and therapeutics. Because methods of molecular biology have only recently been introduced to clinical medicine, they may appear somewhat mysterious and even intimidating to clinicians. Therefore, the purpose of this presentation is to define these methods and describe their impact on clinical medicine. To assist in the reading of this article, a glossary of terms is given in Table 1.

Isolation and Culture of Human and Other Cells

A fundamental step in the development of molecular and cellular biology was the discovery of methods to isolate and replicate in culture cells from humans and other species.[2] This required the development of culture media, specialized glassware and incubators for growing cells. Generally, a cell culture medium consists of a solution of vitamins, minerals and other chemicals to which animal serum is added. Cells can be taken from skin or tumors and replicated in the laboratory. Nonmalignant normal cells will double only 50 or so times. This limitation has been overcome. Some cells such as blood lymphocytes can be infected with virus and "immortalized."[3] The mechanism by which viruses immortalize normal cells is unknown. However, this has the advantage of producing a cell line from any person or animal simply by drawing a tube of blood, isolating the lymphocytes and infecting them with viruses. Because these cells are immortal, they are constantly available for study and can be grown in large quantities.

How is the technique useful? One example is in the study of Huntington's disease. The ability to produce constant cell lines from families in Venezuela with a high incidence of Huntington's disease was very important in DNA studies, which resulted in developing a molecular test for the presence of the gene for Huntington's disease.[4]

Other advances in culturing of cells include techniques for freezing and banking of cells, mass culturing techniques and the development of specialized growth media that allows growth of only cells of interest, such as cancer cells.[5]

Chromosomal Analysis

In the 19th century, it became clear that the behavior of an individual cell was determined by the information contained within the nucleus of the cell. Furthermore, it was discovered that such information was carried by the cell's chromosomes.

A limiting factor during the first part of this century was that it was not clear how many chromosomes humans normally had. Not until 1956 did Tji and Leval definitively show that the normal number of chromosomes in humans was

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46—22 pairs of autosomes and 2 sex-determining chromosomes.[6] In addition, a system for grouping human chromosomes by size was developed. This was an important advance. Appreciation of the normal number of chromosomes and subclassification of chromosomes led to the discovery that certain birth defects such as the Down syndrome were due to an abnormal number of chromosomes.[7]

The next advance in chromosome analysis was techniques for staining chromosomes to identify specific regions or "bands" on the individual chromosomes. Now methods exist for elongating chromosomes so that when band-stained chromosomes are looked at under the microscope, more than 600 regions of bands can be seen on the 46 human chromosomes.[8] This detailed analysis of chromosomes increases the ability to detect chromosomal abnormalities and to relate them to disease.

These newer techniques of chromosomal analysis have been particularly important for the study of cancer. Specific abnormalities consisting of either loss of a portion of a chromosome or translocation of one chromosome region to another chromosome have been reported.[9] For instance, patients with a loss of the p13 region of human chromosome 11 have been found to have an unusually high predisposition to Wilms' tumor of the kidney.[10] Similarly, loss of a region on the long arm of human chromosome 13 has been found to lead to retinoblastoma.[11] Translocation of chromosomes is also seen in malignancy. A chromosome 3 to chromosome 8 translocation is seen in patients with familial renal cell carcinoma.[12] Of course, an abnormal chromosome 22 is familiar in patients with chronic granulocytic leukemia as the so-called Philadelphia chromosome.[13] An intensively studied chromosomal abnormality is the chromosome 8 to 14 translocation of Burkitt's lymphoma.[14] While in the past it was felt by many that these chromosomal alterations were the result of the malignant process, most investigators now feel that they are "signposts" of molecular alterations that are important in the very process of malignant transformation of cells. The translocation of chromosome 8 to chromosome 14 in malignant lymphocytes of Burkitt's lymphoma is thought to unlock the expression of an important oncogene on chromosome 8 because it is placed in proximity to the active immunoglobulin genes of chromosome 14.[15]

Finally, results of microscopic chromosomal analysis using these improved techniques have suggested at least one mechanism by which chromosome breakage, perhaps leading to deletions or translocations, might occur. Scattered throughout our chromosomes are specific sites that are particularly susceptible to breakage, termed "fragile sites."[8] Some of these fragile sites have been found at the chromosomal regions associated with malignancy.[8] It is possible that such

TABLE 1.—*Glossary of Terms in Biomedicine*

Acutely transforming retroviruses
Viruses that have incorporated oncogenes into their structure and as a consequence can malignantly transform cells that they infect

Aneuploidy
An abnormal number of chromosomes

Autoradiography
The detection of radioactively labeled molecules by their effect in creating an image on photographic film

Banding
The staining of human chromosomes with dyes, which allows microscopic delineation of specific regions of chromosomes

cDNA
Complementary DNA derived from reconstructing its genetic code from the protein of messenger RNA for which it codes

Chromosome
The discrete unit of the genome carrying many genes consisting of protein and a very long molecule of DNA visible as a morphologic entity only during the act of cell division

Cloning vectors
Plasmids, cosmids or phage into which genes are inserted for their expansion

Deletion mutant
A cell containing only a fragment of a particular chromosome

DNA [deoxyribonucleic acid]
The chemical component of the cell that encodes the genetic message of the cell

DNA probe
An isolated, expanded DNA sequence that can be used to examine other sequences to determine whether complementary sequences are present, absent or altered

Eukaryotic cells
Cells containing a limiting membrane around their nuclear membrane, as compared with prokaryotes, which do not have such a membrane

Ideotype
The idealized human chromosome karyotype

In situ hybridization
A recombinant DNA technique whereby DNA probes are hybridized directly to intact chromosomes

Kilobase
1,000 bases. A term used to describe the length of DNA molecules; for example, 7.1 kilobases (kb) equals 7,100 bases

Nick translation
The process of labeling, usually with radioisotopes, a DNA probe so that it can be detected

Oncogenes
Gene sequences present within cellular DNA that are able to code for molecular products that cause the malignant transformation of cells; literally, cancer-causing genes

p region
The short arm of a chromosome

q region
The long arm of a chromosome

Recombinant DNA libraries
A collection of cloning vectors containing various portions of genetic material of interest

Reiterated sequence DNA
DNA sequences that are highly prevalent throughout genetic structure but which do not code for specific molecules. Their function is unknown

Restriction
The process of cutting DNA with restriction endonucleases

Restriction site polymorphism
A genetic marker based on the fact that DNA from different persons may be cut into different sizes by a particular restriction endonuclease

Restriction map
The result of separation of DNA fragments in gels after they have been cut with restriction endonucleases

Reverse transcriptase
The enzyme that catalyzes the synthesis of DNA on a template of RNA

RNA [ribonucleic acid]
A macromolecule formed from DNA templates. A particular form of RNA termed messenger RNA (mRNA) carries the active DNA message for protein production.

Somatic cell hybrid
The result of fusion of two different cells (usually from different species) to form a new cell line containing all or part of the genetic information from parent cells

Southern blot
The result of transferring DNA from a gel to filter paper

Unique sequence DNA
DNA sequence coding for a particular protein

sites may provide targets for mutagenic agents, such as radiation. Thus, breakage of the chromosome may be an important step in the development of some malignant processes.

Somatic Cell Hybrids

An important technique for the study of particular chromosomes was the production of somatic cell hybrids.[16] This allows separation of a human chromosome of interest from other human chromosomes. Figure 1 illustrates this process. To form hybrid cells, human cells are fused with rodent cells, usually Chinese hamster or mouse. The fused hybrid cells are then grown under conditions of cell culture that select against parent human and rodent cells and allow growth of only hybrids that contain the human chromosome of interest. The result is a cell that contains the particular chromosome on a background of rodent chromosomes. An important application of this technique has been in gene mapping—that is, the assignment of specific genes to individual chromosomes.[17] Furthermore, it is a mechanism by which chromosomes associated with clinical disorders can be isolated for study. An example is chromosome 21. Production of somatic cell hybrids containing this chromosome has allowed studies of chromosome 21 aimed at identifying the biochemical abnormalities that lead to Down's syndrome.[18]

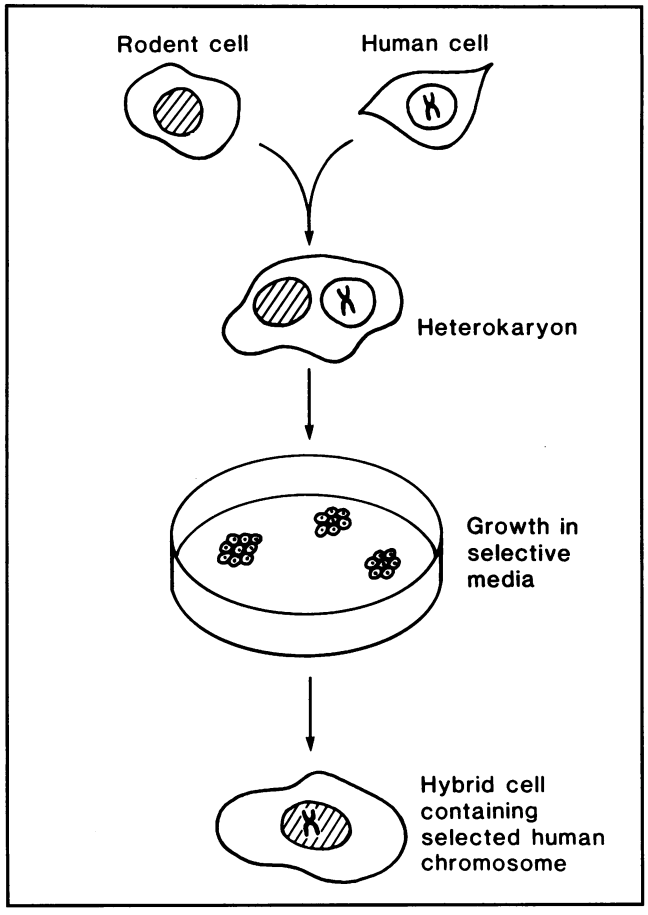

Figure 1.—Production of a somatic cell hybrid. A human cell is fused with a rodent cell using a fusing agent such as a virus or polyethylene glycol. Resulting hybrid cells are grown under conditions of cell culture such that eventually a cell clone is produced that isolates the human chromosome of interest in the rodent cell. (From Scoggin and Fisher.[19])

DNA Isolation

While somatic cell hybrids provide a method whereby individual chromosomes can be isolated, modern techniques of molecular biology allow us to go even further—we can isolate particular genes. An important methodology is isolation and purification of DNA from cells of interest. DNA can also be isolated from the cells in the peripheral blood of patients. Skin biopsies, tumors and cell cultures can also be used. Similarly, DNA can be obtained from other microorganisms such as bacteria, viruses, parasites and mycobacteria. To isolate DNA, cell walls are disrupted enzymatically, protein digested and extracted and the DNA precipitated. This process is well standardized and requires no complexity of equipment beyond a centrifuge. DNA thus purified can be stored where it is readily available for a number of experimental tests.

DNA Separation

To accomplish many of the studies involving DNA, DNA must first be separated into usable pieces. This is done with the use of specialized enzymes called "restriction enzymes" and DNA agarose gel electrophoresis.

Restriction Enzymes

Bacteria possess enzymes that protect them from invasion by foreign DNA. These are restriction enzymes.[20] Many restriction enzymes have been isolated from various bacteria and take their names from the bacteria from which they are isolated. Thus, the enzyme "Eco R1" was isolated from *Escherichia coli (E coli)*. The power of each restriction enzyme is that they recognize and cut DNA at only a specific site in the DNA nucleotide code. For instance, the enzyme Eco R1 will only cut DNA when it sees the nucleotide sequence GAATTC. Restriction enzymes vary in the size of DNA pieces they cut. The enzyme Mbo 1 cuts DNA many more times than Eco R1. To use a very simplistic analogy, using restriction enzymes is somewhat like searching a haystack for a needle. If one wishes to search a haystack, it is helpful to divide the haystack into smaller haystacks. Restriction enzymes accomplish this with DNA. Different restriction enzymes divide DNA into different-sized pieces (or "haystacks"). These enzymes are available commercially and their use is now fundamental in DNA study.

Agarose Gel Electrophoresis

Once the DNA to be studied is cut by restriction enzymes, it must be separated by size, which is accomplished by using agar as a molecular sieve. A gel of agar is poured, the DNA sample to be separated is placed in wells at the top of the gel and a current applied across the gel. This current causes the DNA to move through the gel. The distance a piece of DNA will travel over time is determined by its size. Smaller pieces of DNA will move faster than larger. The result is that the pieces of DNA are separated out throughout the gel, with larger pieces at the top and smaller at the bottom. Fragments of DNA of known size are run as standards parallel with the DNA so that it is known where in the gel DNA samples of a particular size are located. DNA size is expressed in kilobases or kb. Usually DNA is separated from sizes of 50 kb to less than 1 kb. More recently, a technique called "orthogonal field alternation gel electrophoresis" (OFAGE) has been de-

veloped that allows separation of very large pieces of DNA (up to 1,000 kb).[21] This is an important advance because it allows investigation of much larger portions of the genome at a time. This technique has recently been used to determine the actual number of chromosomes contained by the parasite *Plasmodium falciparum*—a necessary step in understanding this important human parasite.[22]

Southern Blotting

Another fundamental technique of DNA research is the Southern blot.[23] Taking its name from E.M. Southern of Edinburgh who developed the technique, it is a method by which DNA is removed from the agarose gel in which it has been separated by size. This is necessary because working with DNA in the gel is very difficult. To accomplish Southern blotting, a piece of nitrocellulose filter paper (or some other matrix such as nylon) is placed over the gel containing the DNA and the DNA transferred onto the paper by drawing a buffer through the gel and filter paper by capillary action. Thus, the DNA is literally blotted onto the filter paper. This process takes 4 to 18 hours. Alternatively, an electric current can be applied across the gel-filter to speed the process. This is termed "electroblotting." DNA transferred to the nitrocellulose or other filter is strongly bound. It represents a replica of the agarose gel, but is much easier to work with. If DNA need not be separated by size, it can be directly applied to the nitrocellulose. This is termed "dot blotting."

To summarize to this point, DNA can be isolated from any nucleated cell; this DNA can be cut into predictable, usable pieces by specialized restriction enzymes. The DNA can then be separated by size by the use of agarose gel electrophoresis. Finally, to facilitate study of the DNA, it is then transferred onto nitrocellulose or other matrix by a process termed Southern blotting.

RNA Isolation and Analysis

Analysis of a messenger RNA (mRNA) provides a method by which genes can be isolated and gene expression studied. Just as the DNA can be isolated from cells, so too can RNA. Additional care must be taken to protect RNA from degradation by ribonuclease enzymes. This is done by adding chemicals or enzymes that inactivate ribonuclease. Messenger RNA must be separated from other types of RNA, which is done by using affinity columns that bind mRNA. Most commonly this is accomplished by a process called "oligo dt chromatography."[24] Isolation of mRNA is important because it can be used to analyze gene expression. Because mRNA is the result of gene expression, it can literally tell investigators which genes are being expressed and in what amounts.

Cloning of Genes

The ability to clone genes basically relies on isolating DNA, cutting DNA with restriction enzymes, cloning vectors to carry DNA, inserting foreign DNA into cloning vectors and using specialized microorganisms for expansion of the cloning vectors containing the "cloned" gene of interest.

Cloning Vectors

Cloning vectors are literally gene carriers. They are plasmids or viruses into which foreign DNA can be inserted. Gene carriers have even been invented, such as cosmids, that allow larger pieces of DNA to be cloned than could be accomplished with virus or plasmids.[26] The availability of restriction enzymes that leave "sticky ends" when they cut DNA has greatly added to the effectiveness of this process of gene cloning.

To clone genes, the cloning vector is cut open with a restriction enzyme that leaves sticky ends. The piece of DNA to be inserted is either cut with the same enzyme to yield similar sticky ends or such sticky ends are attached onto the end of the DNA to be cloned. The DNA to be inserted and the gene carrier are then reacted together and the foreign DNA ligated or "recombined" with the gene carrier. The recombinant vector with the inserted gene is then inserted into a host bacterium that serves as a minifactory in which to grow the gene carrier and its inserted genetic sequence in quantity. By use of selectable markers, such as antibiotic resistance, bacteria containing the gene carrier of interest can be readily identified and isolated. The bacteria used are specialized strains that cannot grow outside of a laboratory environment—a precaution to prevent unnecessary environmental introduction of cloned genes.

DNA for cloning can come from many sources. For instance, it is possible to isolate the messenger RNA coding for a gene of interest and then use an enzyme called "reverse transcriptase" to read the genetic code backwards to produce a complementary DNA (cDNA) sequence of the gene that can then be cloned into a vector such as a plasmid.

All the mRNA from an entire organ, such as liver, can be made into cDNA and cloned into vectors to produce a cDNA "library" of that organ. Such a library would presumably contain the cDNA sequences of all genes expressed by that organ. Thus, the term "library" points out that all the expressed genes have been placed into a collective within cloning vehicles.

Intact DNA itself can also be cloned to form a library. For instance, it is possible to extract the DNA from an entire organ or cell line, cut it with a restriction endonuclease and insert all that DNA into a gene carrier. Because this library would contain all the genetic sequences in the cell, not just those that are expressed as mRNA, it is much more complete than a cDNA library. Such libraries are known as "genomic libraries."[27]

Libraries can even be made of particular chromosomes. For instance, as mentioned earlier, somatic cell hybrids can be used to isolate individual human chromosomes, such as human chromosome 21.[18] A library can be made directly of the DNA from the hybrid. This library will contain both human and hamster sequences—yet this library can be "screened" and the human chromosome members of the library identified and isolated. Alternatively, a specialized machine using lasers can first sort the human chromosome from the hamster chromosomes.[28] The isolated human chromosome DNA can then be used to construct the library.

The ability to clone DNA has been one of the most important advances in molecular biology. It has not only allowed isolation and study of particular genes but has led to industrial methods for producing large amounts of desirable proteins such as insulin. It has also provided a method by which desirable traits can potentially be introduced into food sources. It has also allowed the isolation of genes that appear to be very important in malignant disorders—oncogenes.[29]

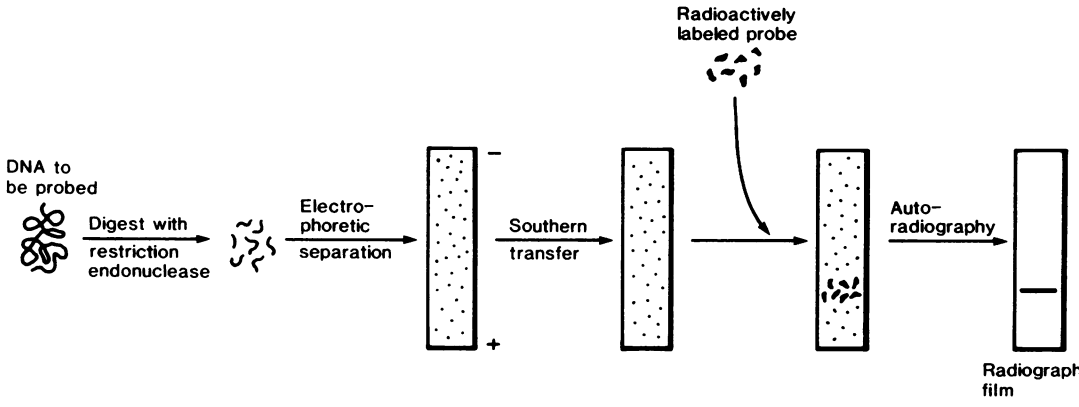

Figure 2.—Southern analysis: DNA to be studied is first digested with a restriction enzyme. DNA is separated by size through agar and then blotted onto nitrocellulose. The Southern blot is then reacted with a radioactive probe. If DNA hybridization has occurred, it will appear as a band of radioactivity when the blot is exposed to radiographic film. (From Scoggin and Fisher.[19])

Gene Analysis

By the use of genetic probes, we are able to peer into the very molecular structure of genes and determine how they are affected by disease. Three important techniques for such analysis are "Southern analysis," "restriction fragment-length polymorphism" and "DNA sequencing."

Southern Analysis

Southern analysis is summarized in Figure 2. As mentioned previously, restriction enzymes cut DNA into useful pieces, agarose gel electrophoresis separates cut DNA by size and Southern blotting removes the DNA from the separation gel onto a membrane surface called a Southern blot. This blot can then be probed with a gene or DNA sequence of interest to find out if the DNA on the blot contains the gene or DNA corresponding to the probe. This is called DNA hybridization and relies on the fact that pairing of nucleotide bases within the double-stranded DNA molecule occurs according to the rules of the genetic code, such that adenine must always pair with thymine and cytosine must only pair with guanine. If the genetic code of the probe does not find its counterpart in the DNA on the Southern blot, hybridization will not occur.

To accomplish such hybridization, the DNA on the Southern blot is rendered single-stranded by treatment with strong alkali. The DNA probe is labeled with radioactivity, or some other tracer, and it is made single-stranded by boiling. Temperature causes DNA double strands to disassociate or "melt" into single strands. The labeled probe and Southern blot are then placed in a plastic bag and allowed to react together, usually overnight. If the probe finds its counterpart on the Southern blot, hybridization will occur. The blot is then washed. Wherever probe has found its complementary DNA sequence, it will not be washed off. If radioactivity has been used as a probe's label, when the Southern blot is exposed to radiographic film, exposure will occur and a "band" will be seen on the film. Because disease will sometimes alter the size of a gene or where a restriction enzyme cuts the gene, not only is it important to know whether or not a gene is present in a DNA sample, but also its size. Both these pieces of information can be determined by Southern analysis.

Restriction Fragment-Length Polymorphism

Restriction fragment-length polymorphism is a somewhat formidable term for what is really a rather simple but important concept. It allows construction of genetic maps of DNA and correlation of such maps with particular traits—a recent example being Huntington's disease.[4]

A polymorphism is the coexistence of more than one variant of a particular trait. A restriction fragment-length polymorphism refers to the fact that the DNA from any particular region in different persons may be cut into different-sized pieces by the same restriction enzymes. This will result in bands of DNA on a Southern blot appearing at different levels. This type of blot is a "restriction map." Polymorphisms in the genome can be detected by comparing the restriction maps of different persons. A difference in restriction maps between two persons can be used like any other genetic marker. The only difference is that the DNA itself is examined at the molecular level and not somatic phenotypic markers such as blood group or eye color. It is an extremely powerful technique. In the future, a genetic map of the human genome will probably be constructed that will allow detection of carrier and affected persons with such important disorders as cystic fibrosis. Restriction fragment-length polymorphism also allows identification of the particular chromosomal region that carries the genes responsible for the disorder—an important step in understanding the molecular basis of any disease.

DNA Sequencing

Not only can DNA be isolated and characterized by size and restriction pattern, the actual sequences of the base pairs that make up DNA can also be ascertained. This is literally "decoding" DNA. Protocols are now available by which DNA can be either chemically cleaved or synthesized in such a way that at each step the involved nucleotide base sequentially affected is known.[30] Step by step the sequence of the DNA of interest is determined. This is now such an accurate procedure that DNA molecules of 300 base pairs or even larger are more easily analyzed than the proteins sequenced for which they code. With the use of computers, DNA sequences of various genes are being made widely available for scientific comparison. This sequence information has also allowed the development of technology to synthetically produce genes or parts of genes.

Summary

While in the past it has been rightly said that basic science and clinical medicine are on a collision course, it can now be legitimately stated that the collision has occurred. Molecular biology has given us a whole new microscope with which to

study human disease and every other type of biologic phenomenon. Southern blots showing the basic investigation of such clinical disorders as cancer and infection are now regularly seen in medical journals. Useful genes such as those for insulin and growth hormones have been cloned and their products made available as pharmaceutic agents.[31] Other genes are also being isolated for production—such as α_1-antitrypsin for persons with congenital deficiencies of the enzymes and premature emphysema.[32] Experiments are even being conducted to change the site on the α_1-antitrypsin molecule that is subject to biologic degradation. This involves a process called "site-directed mutagenesis," which induces a change in the genetic code for the enzyme.[32] If it is successful, it would be somewhat like instant evolution to produce a more effective enzyme than what would normally occur. DNA probes for infectious diseases are also being introduced into the clinical laboratory.[33] The result will be faster, cheaper and more accurate tests.

DNA hybridization has provided improved tests for such hereditary disorders as sickle cell anemia and thalassemia.[34-36] Diagnostic tests for other important hereditary disorders such as Huntington's disease and cystic fibrosis are being developed or sought. The ultimate payoff, however, is not going to be just improved diagnostic and pharmaceutical production. While an important advance, producing human insulin does not cure diabetes. The reward of this new technology will be in the understanding and methodologies that are to come that will allow entirely new strategies for directly correcting the metabolic malfunction of disease.[37] It is reasonable to speculate that in the future, physicians will be able to intelligently manipulate the biochemistry of the body in a way analogous to how a surgeon now manipulates organ function.

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